Analysis of microsatellite instability in medulloblastoma

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Medulloblastoma is the most common malignant brain tumor in children. The presence of microsatellite instability (MSI) in brain tumors, particularly medulloblastomas, has not been properly addressed. The aim of the present study was to evaluate the role of MSI in medulloblastoma carcinogenesis. MSI status was determined in 36 patients using a pentaplex PCR of quasimonomorphic markers (NR27, NR21, NR24, BAT25, and BAT26). Methylation status of mismatch repair (MMR) genes was achieved by methylation-specific multiplex ligation-dependent probe amplification (MLPA). In addition, MutS homolog 6 (MSH6) expression was determined by immunohistochemistry. Mutations of 10 MSI target genes (*TCF4*, *XRCC2*, *MBD4*, *MRE11*, *ATR*, *MSH3*, *TGFBR2*, *RAD50*, *MSH6*, and *BAX*) were studied by pentaplex PCR followed by analysis with GeneScan 3.7 software. Mutation analysis of hotspot regions of *β-catenin* (*CTNNB1*) and *BRAF* (*v-raf murine sarcoma viral oncogene homolog B1*) oncogenes was performed by PCR single-strand conformation polymorphism analysis followed by direct sequencing. Among the 36 tumors, we found four (11%) cases with instability, one with high MSI and three with low MSI. Methylation analysis of MMR genes in cases presenting shifts on the MSI markers revealed mild hypermethylation of *MSH6* in 75% of cases, yet MSH6 was expressed in all the tumors. The MSI target genes *MBD4* (*methyl-CpG binding domain protein 4*) and *MRE11* (*meiotic recombination 11 homolog A*) were mutated in two different tumors. No *CTNNB1* or *BRAF* mutations were found. This study is the most comprehensive analysis of MSI in medulloblastomas to date. We observed the presence of MSI together with mutations of MSI target genes in a small fraction of cases, suggesting a new genetic pathway for a role in medulloblastoma development. *Neuro-Oncology 11, 458–467, 2009 (Posted to Neuro-Oncology [serial online], Doc. D08-00196, January 29, 2009. URL http://neuro-oncology.dukejournals.org; DOI: 10.1215/15228517-2008-115)*

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Brain tumors are the second most common malignancy among children and the leading cause of cancer-related death in pediatric patients. The overall incidence rate for pediatric brain tumors (occurring from birth through 19 years) is 4.3 per 100,000 person-years.[1] Among the different histological entities, medulloblastoma is the most common childhood malignant brain tumor, accounting for approximately 20% of all pediatric intracranial tumors, with a peak incidence between 3 and 4 years of age.[1] Medulloblastomas are less common in adults, with a peak incidence between 20 and 35 years.[2] Current therapy for this malignancy is very aggressive, including maximum surgical resection, craniospinal radiotherapy, and adjuvant chemo-

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therapy, yet the medulloblastoma 5-year survival rate is only 50%–60%,[3,4] and these aggressive procedures frequently have serious neurocognitive and endocrine sequelae in survivors, particularly in younger patients.[5]

Genetic instability is a paramount feature of cancer, which leads to accumulation of genetic alterations that varies from subtle changes in DNA sequence to chromosomal abnormalities.[6] Microsatellite instability (MSI) is a particular type of genetic instability affecting short sequences of DNA repeats (microsatellites) found throughout the genome.[6] MSI was first described in hereditary nonpolyposis colorectal cancer (HNPCC) and is present in the majority of these patients; currently, MSI analysis of this malignancy is standardized by the Bethesda guidelines.[7] In colorectal cancer (CRC), the MSI phenotype appears to be related to particular clinical and histopathological features, including location in the proximal colon, tumors poorly differentiated with mucinous and signet ring cells, high tumor lymphocyte infiltration, low frequency of distant metastasis, and a comparably good prognosis.[8] The MSI phenotype is a consequence of deficient DNA mismatch repair (MMR), which fails to recognize errors introduced in microsatellite regions during DNA replication. The loss of function of MMR family genes (*MLH1*, *MLH3*, *MSH2*, *MSH3*, *MSH6*, *PMS1*, and *PMS2*) is caused by germline mutations in hereditary malignancies, whereas in sporadic cancers, *MLH1* (*mutL homolog 1, colon cancer, nonpolyposis type 2*) promoter methylation has been shown to be the main cause of gene silencing.[9] As naturally occurring replication errors are not efficiently repaired, tumors with MMR deficiencies have a higher number of nucleotide insertions/deletions in genes harboring microsatellites.[6,9] The accumulation of activating or inactivating frameshift mutations in genes that regulate cell functioning, such as *TGFBR2* (*transforming growth factor* β *type II receptor*) and *BAX* (*BCL2-associated X protein*), is thought to be responsible for the tumorigenic process of MSI in MMR-deficient cells.[9] Particularly important to oncological research is the evidence that many of these mutated genes, already identified in different tumors, also appear to have a role in the therapeutic response of different anticancer drugs.[10–12]

Previous studies have evaluated the presence of MSI in brain tumors, mainly gliomas. An absence or a rare incidence of MSI in adults and contradictory results in pediatric patients have been reported.[13–21] In medulloblastoma, MSI status has not been properly characterized.

The aim of the present study was to evaluate the presence of MSI in medulloblastomas, using a panel of markers recommended by the revised Bethesda guidelines.[7] In addition, in tumors presenting MSI, we assessed the molecular status of MMR genes and the mutation profiles of 10 potential MSI target genes (*TCF4*, *XRCC2*, *MBD4*, *MRE11*, *ATR*, *MSH3*, *TGFBR2*, *RAD50*, *MSH6*, and *BAX*). Furthermore, we analyzed mutations of *BRAF* (*v-raf murine sarcoma viral oncogene homolog B1*) and *β-catenin* (*CTNNB1*).

Material and Methods

Patients and Tumor Samples

Formalin-fixed, paraffin-embedded samples from 36 cases of medulloblastoma were retrieved from the Pathology Department of Santa Maria Hospital, Lisbon, Portugal. Tumor samples were classified according to WHO criteria.[22] Thirty-four of the 36 cases were classic medulloblastomas, and the remaining two were classified as desmoplastic medulloblastomas. Of the patients, 22 (61.1%) were male and 14 (38.9%) were female; the mean age was 19.5 years (range, 1.5–70 years; Table 1).

DNA Extraction

DNA was extracted from 10-μm-thick formalin-fixed, paraffin-embedded tumor samples as previously described.[23] Briefly, tissues were deparaffinized by a serial extraction with xylene and ethanol (100%/70%/50%), and separately selected areas of tumor and normal tissue, when available, were microdissected using a sterile needle and carefully collected into a 0.2-mL PCR tube. DNA was extracted using QIAamp DNA Micro Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

MSI Analysis

The MSI evaluation was performed using a multiplex PCR comprising five quasimonomorphic mononucleotide repeat markers (NR27, NR21, NR24, BAT25, and BAT26).[24] Primer sequences were described previously.[24] Each antisense primer was end-labeled with 6-carboxyfluorescein (FAM), hexachloro-6-carboxyfluorescein (HEX), or tetrachloro-6-carboxyfluorescein (TET) fluorescent marker. PCR was performed using the Qiagen Multiplex PCR Kit, and then products were separated using an ABI Prism 310 single capillary genetic analyzer (Applied Biosystems, Foster City, CA, USA). The MSI status of the tumor was analyzed using GeneScan analysis software (version 3.7; Applied Biosystems). Cases exhibiting instability at two or more markers were considered to have high MSI (MSI-H), those with instability at one marker were defined as having low MSI (MSI-L), and those showing no instability were defined as microsatellite stable (MSS), as previously described.[25] DNA from the cell lines HCT15 (MSI) and DAOY (MSS) were used as positive and negative controls, respectively. The quasimonomorphic variation range of each marker (described by Buhard et al.[26]) was established in our analysis using a series of DNA from six healthy people.

Mutation Analysis of MSI Target Genes

Selected genes containing repeated sequences previously described as frequent targets for instability were chosen for frameshift mutation study by fragment analysis and further genomic sequencing confirmation. The selected

Table 1. Detection of microsatellite instability (MSI) in medulloblastomas using five mononucleotide repeat markers

Case	Gender/Age (Years)	Histological Type	MSI Detection					
			NR27	NR21	NR24	BAT25	BAT26	MSI State
M1	M/11	Classic	86	106	123	148	179	MSS
M2	F/16	Classic	86	107	123	148	180	MSS
M3	M/35	Classic	87	107	122	148	180	MSS
M4	M/33	Classic	86	107	123	148	180	MSS
M5	M/1.5	Classic	86	**102–107**	122	148	179	MSS
M6	M/10	Classic	86	107	123	147	180	MSS
M7	F/70	Classic	86	**102–106**	123	147	180	MSS
M8	M/20	Classic	87	106	122	148	180	MSS
M9	F/26	Classic	86	**102–107**	123	147	180	MSS
M10	F/10	Classic	86	106	123	147	180	MSS
M11	F/1.5	Classic	87	106	123	148	180	MSS
M12	F/1.5	Classic	87	106	123	148	180	MSS
M13	M/21	Desmoplastic	**84**	105	122	146	**168**	**MSI-H**
M14	M/11	Classic	86	107	122	148	179	MSS
M15	M/28	Classic	87	107	123	146	179	MSS
M16	M/34	Classic	86	107	122	148	179	MSS
M17	M/6	Classic	86	106	123	148	180	MSS
M18	F/24	Classic	86	106	122	147	180	MSS
M19	F/34	Classic	87	106	123	148	179	MSS
M20	M/12	Classic	86	106	123	147	180	MSS
M21	M/10	Classic	86	105	122	**147–150**	179	**MSI-L**
M22	F/16	Classic	86	106	123	148	180	MSS
M23	M/8	Classic	86	107	123	148	NA	MSS
M24	M/7	Classic	87	106	123	148	180	MSS
M25	M/35	Classic	87	107	123	**144–148**	179	**MSI-L**
M26	M/2	Classic	86	106	123	147	180	MSS
M27	M/65	Classic	86	107	122	148	180	MSS
M28	M/39	Classic	86	107	123	147	180	MSS
M29	F/8	Classic	87	107	122	147	179	MSS
M30	F/26	Classic	86	106	122	148	179	MSS
M31	F/2	Classic	86	107	122	146	180	MSS
M32	M/33	Classic	86	106	122	147	180	MSS
M33	M/4	Classic	86	107	123	148	180	MSS
M34	F/9	Classic	86	107	123	147	180	MSS
M35	M/13	Classic	**84**	106	122	146	179	**MSI-L**
M36	F/18	Desmoplastic	86	106	123	148	180	MSS

Abbreviations: M, male; MSS, microsatellite stability; F, female; MSI-H, high microsatellite instability; MSI-L, low microsatellite instability; NA, not amplified. Boldface indicates MSI markers presenting alterations.

genes were *transcription factor-4* (*TCF4*; poly[A]9), *X-ray repair cross-complementing protein 2* (*XRCC2*; T8), *methyl-CpG binding domain protein 4* (*MBD4*; A10), *meiotic recombination 11 homolog A* (*MRE11*; T11), *ataxia telangiectasia and Rad3 related checkpoint kinase 1* (*ATR*; A10), *MSH3* (A8), *TGFBR2* (A10), *RAD50 homolog* (*RAD50*; A9), *MSH6* (C8), and *BAX* (G8).[27,28] PCR was performed with primers, end-labeled with FAM, HEX, or TET fluorescent markers, specific for each selected candidate gene, as previously described.[27,28] PCR products were separated using an ABI Prism 310 single capillary genetic analyzer (Applied Biosystems), and the PCR products profiles were analyzed using GeneScan 3.7 software (Applied Biosystems). Several normal DNA samples were used to establish profile patterns for each gene, and mutation analysis was performed comparing the peak pattern alterations with the reference peak size and pattern.[27,28] Analyses of samples presenting abnormal profiles were repeated three times by multiplex and monoplex PCR. In addition, PCR followed by direct sequencing was performed to confirm the presence of a frameshift mutation.

Mutation Analysis of CTNNB1 and BRAF Oncogenes

Screening of hotspot mutations on *CTNNB1* exon 3 and *BRAF* exon 15 was carried out by PCR single-strand conformation polymorphism as previously described.[29–31]

Methylation Analysis of the MMR System Genes

The study of *MLH1*, *MLH3*, *MSH2*, *MSH3*, *MSH6*, and *PMS2* (*PMS1 postmeiotic segregation increased 2*) MMR gene methylation was performed by methylation-specific MLPA kit ME011 according to the manufacturer's instructions (MRC-Holland, Amsterdam, The Netherlands).[32] Briefly, 100 ng tumoral DNA was denaturated in 5 µl ultrapure water at 98°C for 5 min and then incubated with the probe mix for 20 h at 60°C. After probe hybridization, each sample was divided into two tubes. Half of the sample was ligated using a ligase enzyme, and in the other half ligation was combined with HhaI digestion, resulting in ligation of the methylated sequences only. The resulting products were amplified by PCR using a FAM-labeled primer following manufacturer's instructions. PCR products were analyzed on an ABI Prism 310 single capillary genetic analyzer (Applied Biosystems) using GeneScan 3.7 software (Applied Biosystems). Duplicate experiments were performed for methylation analysis, and average ratios were calculated. Additionally, the overall average of the different probes of the same gene was calculated. Data analysis was performed as described by the manufacturer. We interpreted (average) ratios as absence of hypermethylation (0.00–0.24), mild hypermethylation (0.25–0.49), moderate hypermethylation (0.50–0.74), and extensive hypermethylation (≥0.75), as previously described.[33]

Immunohistochemistry to the MSH6 MMR System Protein

Immunohistochemistry analysis of MSH6 protein was performed using 3-µm paraffin-embedded tissue sections. Tissue sections were deparaffined, rehydrated in graded ethanol, and washed. Antigen retrieval was achieved by microwave treatment in 1 mM EDTA (pH 8.0) for 15 min. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2. To block nonspecific protein binding, sections were incubated with R.T.U. normal horse serum (R.T.U. vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). A primary antibody to MSH6 (clone 44, purified mouse anti-MSH6; BD Transduction Laboratories, BD Biosciences, Erembodegem, Belgium) was applied at a concentration of 1:100 and incubated overnight at room temperature. Antigen–antibody complexes were revealed by a 10-min incubation with R.T.U. biotinylated universal antibody antirabbit/mouse IgG (H + L) (R.T.U. vectastain Elite ABC kit; Vector Laboratories) followed by incubation with R.T.U. vectastain Elite ABC reagent (R.T.U. vectastain Elite ABC kit; Vector Laboratories) for 10 min 3,3/-Diaminobenzidine (Dako Liquid DAB, DakoCytomation, VitaReal, Carpinteria, CA, USA) was used as a chromogen. Slides were counterstained with hematoxylin. Normal colon tissue was used as a positive control. A negative control was also used (DakoCytomation N-Universal Negative Control Mouse, DakoCytomation).

Results

MSI Analysis

MSI analysis was performed for 36 tumors using a pentaplex PCR of quasimonomorphic markers recommended by the revised Bethesda guidelines.[7] Among all samples, we found four (11.1%) cases with instability—one with MSI-H and three with MSI-L—and 32 MSS (88.9%) (Table 1, Fig. 1). Regarding adult and pediatric patients, we found 13% (2 of 15) MSI tumors (M13, M25) in the adult set, one of which was MSI-H (M13). In pediatric samples, two cases (9.5%) were MSI-L (M21, M35). Case M13 presented mutations in NR27 and BAT26 markers, cases M21 and M25 presented alterations in BAT25, and M35 presented alterations in NR27. Three cases (M5, M7, and M9) presented allele variants in NR21. Case M13 had adjacent normal DNA available, which did not exhibit the alterations present in the tumor DNA (Fig. 1). In addition, the MSI status of both tumor and normal DNA was confirmed by direct sequencing.

MMR Gene Methylation

Promoter abnormal methylation of MMR genes is the main mechanism underlying MSI phenotype in sporadic tumors. Therefore, we analyzed methylation of the main

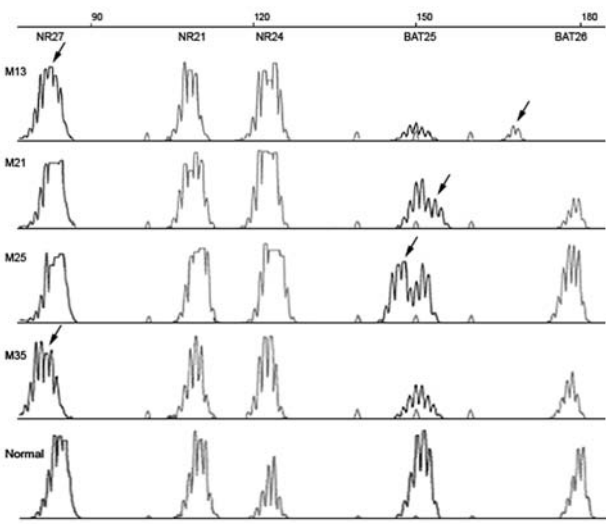

Fig. 1. Altered markers in the microsatellite instability cases: pentaplex PCR with the microsatellite quasimonomorphic markers (NR27, NR21, NR24, BAT25, and BAT26). Case M13 presented mutation in NR27 and BAT26 microsatellite markers, cases M21 and M25 were mutated in the BAT25 marker, and M35 in NR27 (altered markers indicated with arrows).

Table 2. Methylation status of mismatch repair (MMR) genes in medulloblastomas

		MMR Gene Methylation (Average)					
Case	MSI Status	MLH1	MLH3	MSH2	MSH3	MSH6	PMS2
M5	MSS	U (0.15)	U (0.05)	U (0.08)	U (0.17)	U (0.19)	U (0.10)
M7	MSS	U (0.05)	U (0.02)	U (0.07)	U (0.19)	U (0.16)	U (0.11)
M9	MSS	U (0.11)	U (0.00)	U (0.05)	U (0.17)	**M (0.27)**	U (0.09)
M13	MSI	U (0.15)	U (0.06)	U (0.11)	U (0.20)	**M (0.29)**	U (0.13)
M21	MSI	U (0.11)	U (0.18)	U (0.06)	U (0.23)	**M (0.26)**	U (0.11)
M25	MSI	U (0.10)	U (0.00)	U (0.05)	U (0.15)	U (0.23)	U (0.07)
M35	MSI	U (0.02)	U (0.08)	U (0.08)	U (0.00)	U (0.12)	U (0.07)

Abbreviations: MSI, microsatellite instability; *MLH1 and MLH3*, *mutL homolog 1, colon cancer, nonpolyposis, types 2* and *3*; *MSH2*, *MSH3*, and *MSH6*, *MutS homologs 2*, *3*, and *6*; *PMS2*, *PMS1 postmeiotic segregation increased 2*; MSS, microsatellite stability; U, unmethylated; M, methylated. Boldface indicates the presence of gene methylation.

MMR genes (*MLH1*, *MLH3*, *MSH2*, *MSH3*, *MSH6*, and *PMS2*) in the MSI-H tumor (M13), in three tumors presenting MSI-L (M21, M25, and M35), and in the three additional cases presenting an allele variant in the NR21 marker (Table 2). Except for *MSH6*, none of the other MMR genes showed promoter gene hypermethylation. *MSH6* presented mild hypermethylation in 43% (three of seven) of the cases analyzed: in two MSI (M13 and M21) and one MSS (M9).

Immunohistochemistry of the MSH6 MMR System Protein

The immunohistochemical assay was performed to complement the methylation study of *MSH6* and to understand the effects of mild gene hypermethylation on protein expression levels. All cases exhibited MSH6 positivity, but cases M5 and M25 showed weaker staining (Fig. 2).

Mutation Analysis of MSI Target Genes

Selected MSI target genes (*TCF4*, *XRCC2*, *MBD4*, *MRE11*, *ATR*, *MSH3*, *TGFBR2*, *RAD50*, *MSH6*, and *BAX*) were analyzed for frameshift mutations in the MSI tumors and in the cases presenting an allele variant in the NR21 marker (Table 3). Among the genes studied, *MBD4* and *MRE11* have been shown to be mutated in one MSI sample each (1 of 4 = 25%) in M13 (MSI-H) and M21 (MSI-L). Both *MBD4* and *MRE11* had a heterozygous insertion of one base pair. Therefore, two of the four tumors with instability presented mutation in one MSI target gene. The presence of frameshift mutations was confirmed by direct sequencing and demonstrated to be heterozygous by both techniques (Figs. 3 and 4).

Mutation Analysis of CTNNB1 *and* BRAF *Oncogenes*

Because the Wingless/Wnt signal transduction pathway is involved in medulloblastoma development, we searched for mutations in its critical downstream effector *CTNNB1*. All cases were analyzed for mutations in exon 3. No medulloblastoma showed any *CTNNB1* genetic alteration.

BRAF mutations, particularly the V600E hotspot mutation, have been described to be involved in colorectal carcinomas exhibiting MSI. None of the MSI medulloblastomas exhibited *BRAF* exon 15 mutations.

Discussion

MSI was first identified in and is present in about 90% of HNPCC cases.[34] This phenotype has also been described in many sporadic human malignancies and is present in approximately 10%–15% of colorectal, endometrial, and gastric cancers.[34] The few studies reporting MSI status in brain tumors showed that this phenotype is a rare event (0%–8%) in adult sporadic CNS tumors.[13–17,19] Regarding pediatric data, the results are contradictory: MSI was found in 0%–27% of CNS tumors studied.[13–16,18–21] In the present study, we screened 21 pediatric and 15 adult medulloblastomas for MSI. The overall incidence of instability was 11% (4 of 36 cases), with three cases showing MSI-L and a single case with MSI-H, from two adults and two pediatric patients. According to these results, the presence of MSI in medulloblastomas appears not to be age-related, in contrast to data from other CNS tumors. The few studies that analyzed MSI status in medulloblastomas reported the absence of genetic instability.[15,21,35] However, these studies evaluated a very small number of cases, some not differentiating medulloblastomas from other primitive neuroectodermal tumors,[15,21] using panels of microsatellite markers that were limited for MSI status assessment.[16,17,21] In this work, we used a gold standard panel of microsatellite markers recommended by the revised Bethesda guidelines for CRC.[7] This panel of mononucleotide markers provides high specificity and sensitivity for MSI detection, and their quasimonomorphic nature allows the analysis of MSI status without the need to evaluate corresponding normal tissue.[24]

The main mechanism driving MSI in sporadic tumors has been shown to be methylation of MMR genes. We analyzed the methylation of the *MLH1*, *MLH3*, *MSH2*, *MSH3*, *MSH6*, and *PMS2* MMR genes in tumors with MSI and in those presenting an allelic variant of the NR21 marker but considered MSS, and found mild

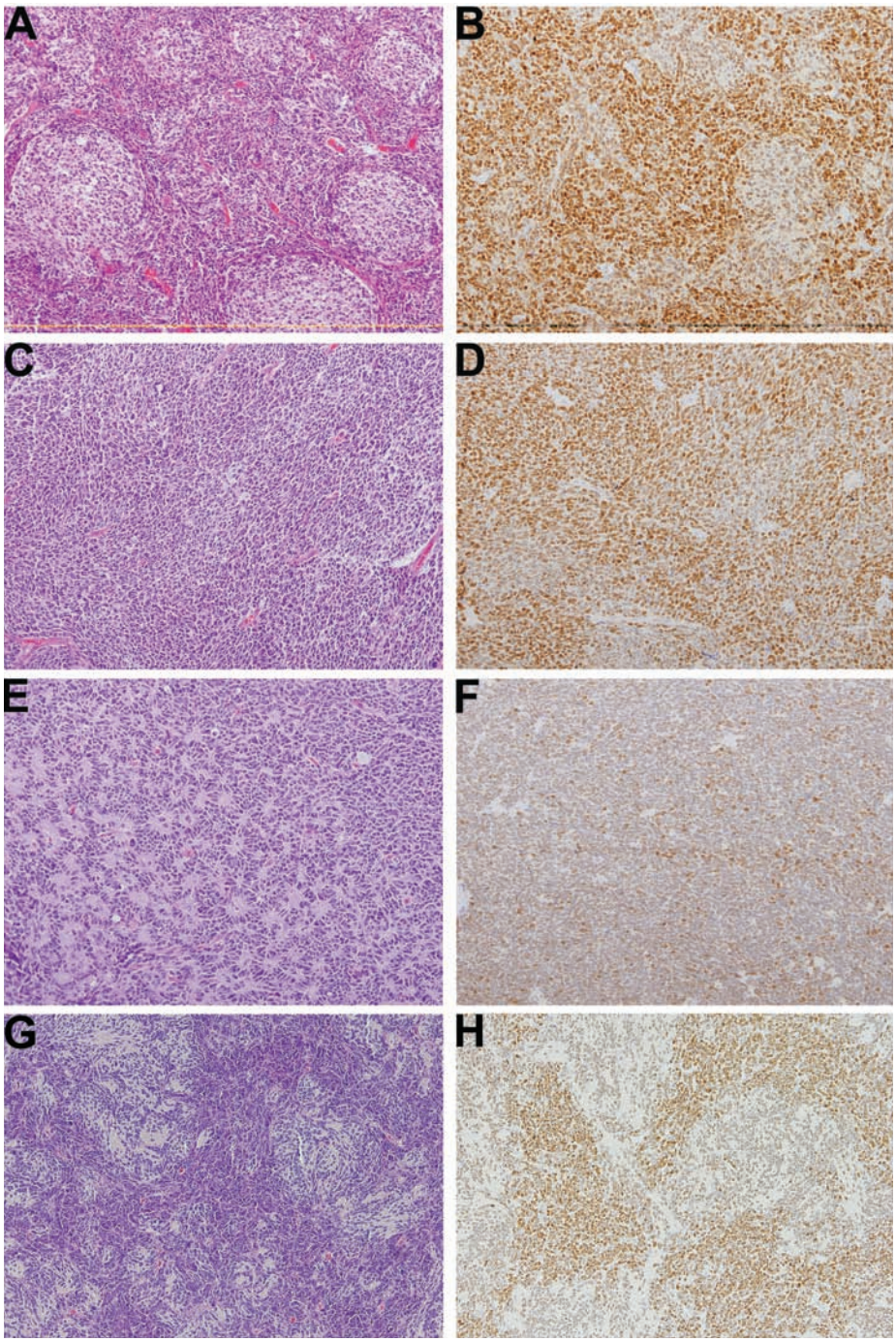

Fig. 2. Representative histology and immunohistochemistry results: hematoxylin and eosin (A, C, E, and G) and MutS homolog 6 (MSH6) immunohistochemistry (B, D, F, and H) for the microsatellite-instability–positive medulloblastomas M13 (A and B), M21 (C and D), M25 (E and F), and M35 (G and H). Original magnification: ×100.

hypermethylation levels of the *MSH6* gene in two cases of MSI (M13 and M21) and one case of MSS (M9). Nevertheless, the presence of mild hypermethylation was not transduced in a lack of protein expression and might not be the cause of the MSI phenotype. It remains to be determined in MSI-positive medulloblastomas which MMR protein is affected and by which mechanism. The presence of MMR deficiencies is well correlated with the MSI status in several tumors, such as CRC,[36] ovarian carcinoma,[37,38] and endometrial carcinoma,[38,39] where MSI-H tumors present inactivation of MMR proteins whereas the genes leading to MSI-L are unclear.[7,40] However, in other tumor entities such as Ewing tumors, such associations were not reported.[41] This suggests that MMR protein deficiencies in MSI-positive tumors depend not only on MSI levels but also on the tumor

Table 3. Candidate microsatellite instability target gene frameshift mutations detected in medulloblastomas

Case	TCF4 (A9)	XRCC2 (T8)	MBD4 (A10)	MRE11 (T11)	ATR (A10)	MSH3 (A8)	TGFBR2 (A10)	RAD50 (A9)	MSH6 (C8)	BAX (G8)	Mutation Frequency
M5	A	A	A	A	A	A	A	A	A	A	0 of 10
M7	A	A	A	A	A	A	A	A	A	A	0 of 10
M9	A	A	A	A	A	A	A	A	A	A	0 of 10
M13	A	A	A	**P** (T11–12)	A	A	A	A	A	A	1 of 10
M21	A	A	**P** (A10–11)	A	A	A	A	A	A	A	1 of 10
M25	A	A	A	A	A	A	A	A	A	A	0 of 10
M35	A	A	A	A	A	A	A	A	A	A	0 of 10
Tumors with mutation	0 of 7	0 of 7	1 of 7	1 of 7	0 of 7	0 of 7	0 of 7	0 of 7	0 of 7	0 of 7	

Abbreviations: *TCF4, transcription factor-4*; *XRCC2, x-ray repair cross-complementing protein 2*; *MBD4, methyl-CpG binding domain protein 4*; *MRE11, meiotic recombination 11 homolog A*; *ATR, ataxia telangiectasia and Rad3 related checkpoint kinase 1*; *MSH3, MutS homolog 3*; *TGFBR2, transforming growth factor β type II receptor*; *RAD50, RAD50 homolog*; *MSH6, MutS homolog 6*; *BAX, BCL2-associated X protein*; A, absent; P, present.

type. Data on MMR gene alterations are scarce in CNS tumors. In medulloblastoma, only one study has examined MMR protein expression.[42] The authors reported the absence of any deficiency in MLH1, MSH2, and PMS2 proteins in a series of 22 medulloblastomas.[42]

In order to evaluate the mutagenic effect of the MSI phenotype in medulloblastomas, we performed a mutation analysis of candidate MSI target genes. We studied 10 candidate genes—*TCF4*, *XRCC2*, *MBD4*, *MRE11*, *ATR*, *MSH3*, *TGFBR2*, *RAD50*, *MSH6*, and *BAX*—previously described to be frequently mutated in MSI tumors such as colorectal, urothelial, or endometrium cancers.[27,28,43] Most of the candidate target gene mutations were primarily found and mainly analyzed in MSI-H CRC. Although several of these mutations have already been reported in different MSI-H tumors, this is not true for all different MSI cancers. We found alterations in *MBD4* and *MRE11* genes in two of the four MSI

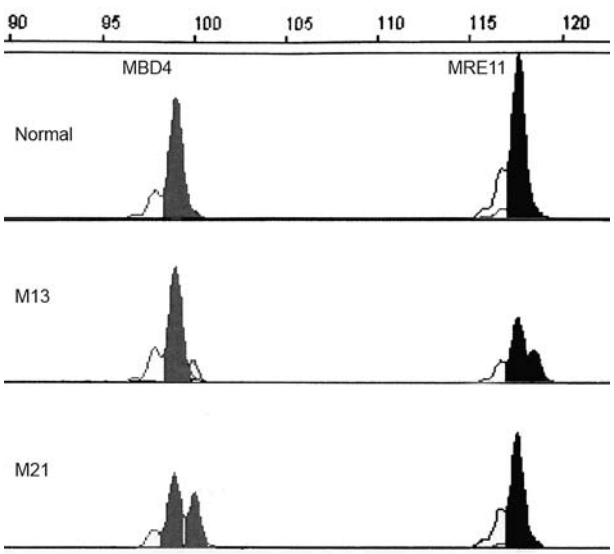

Fig. 3. Microsatellite instability target gene mutations: fragment analysis of *methyl-CpG binding domain protein 4* (*MBD4*) and *meiotic recombination 11 homolog A* (*MRE11*) for normal DNA from a healthy person, and mutated fragments by insertion of one nucleotide in MRE11 in case M13 and in MBD4 in case M21.

medulloblastomas, one with MSI-H and one with MSI-L. Overall, among the 10 candidate target genes studied, this represents 20% of genes mutated. This rate of mutation in candidate target genes, although smaller than what is regularly stated for CRC, is comparable to frequencies reported for other tumor entities such as pancreatic ductal adenocarcinomas, which presents 25% mutated genes,[44] and it is higher than the incidence reported in other studies,[45] indicating that different tumors can present mutations in different targets for instability.[46,47] In addition, as mentioned above, mutations in candidate target genes were mainly reported only in MSI-H tumors, in different tumor entities.[44,48] Among these different cancers, MSI-L malignancies generally do not present mutations in the candidate target genes frequently mutated in MSI-H tumors, but our results raise the question of whether this is true for all MSI-L tumors.

MBD4 is a member of the methyl-CpG binding protein family, which possesses a methyl-CpG binding domain (MBD) and a glycosylase repair domain, repairing mismatched G-T residues at methylated CpG sites.[49] Previous studies in colorectal, endometrial, and gastric tumors reported that truncating mutations of *MBD4*, due to the deletion of one nucleotide in the A10 tract of exon 3, result in proteins without the glycosylase repair domain and therefore with defective glycosylase activity.[49–51] In addition, MBD4 truncated protein had the capacity to compete with wild-type protein in a dominant negative manner, causing the accumulation of errors in the DNA.[51] In our study we observed not a deletion but a nucleotide insertion at the A10 tract of the *MBD4* gene. Similar to deletion, insertion of a nucleotide in the A10 microsatellite region is also suggested to result in a truncated and defective protein.[50]

MRE11 is a member of the MRE11/NBS1/RAD50 (MNR) complex, which is essential for the maintenance of DNA integrity. This complex plays a central role in recognizing and repairing double-strand breaks through homologous recombination or nonhomologous end-joining repair pathways. It was previously suggested that homo- or heterozygous deletions in the poly(T)11 within *MRE11* intron 4 cause aberrant splicing, with skipping of exon 5, leading to a premature stop codon and generation of a truncated protein.[52] In this study,

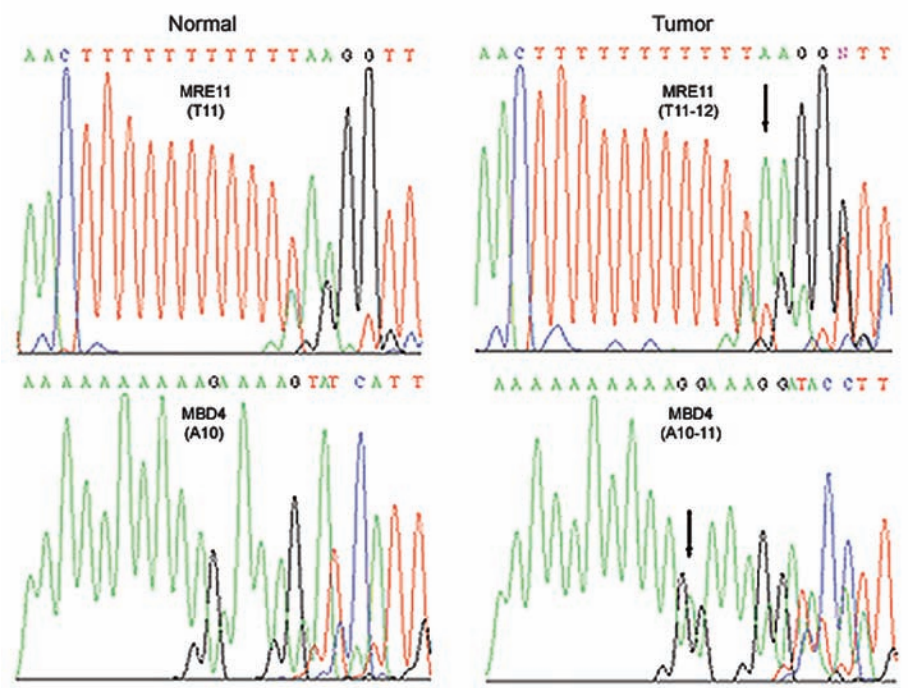

Fig. 4. DNA sequencing of the microsatellite instability target genes *meiotic recombination 11 homolog A* (*MRE11*; T11) and *methyl-CpG binding domain protein 4* (*MBD4*; A10): normal DNA from a healthy person for the *MRE11* and *MBD4* genes (left) and the mutated cases M13 for *MRE11* and M21 for *MBD4* (right). Arrows indicate the insertion of one nucleotide.

we observed a heterozygous insertion in *MRE11* T11 in case M13. This allele expansion was also identified in a CRC tumor sample of a Lynch syndrome patient, and can also result in aberrant splicing signals and premature stop codons of *MRE11*.[52] Nevertheless, the exact functional role of the poly(T)11–12 *MRE11* mutation is not clear, and the same mutation was also reported in a lymphoma cell line.[53]

Although the majority of medulloblastomas occur sporadically, they can occur associated with Turcot syndrome type 2,[22] and it also has been reported in young members of families with Lynch syndrome.[54–56] Interestingly, biallelic germline mutations in *MSH6*[54,55] or *MLH1*[56] were described in three different patients that developed medulloblastoma. Tumors presenting *MSH6* mutations were also found to lack protein expression,[54,55] whereas in medulloblastoma with *MLH1* mutation, protein expression was not reported.[56] Despite due diligence, it was not possible to obtain the family history of the four patients with MSI medulloblastoma to assess their potential inherited nature. Therefore, we cannot exclude the possibility that these MSI-presenting tumors arose in a familial cancer context. Aiming to unravel this question, somatic *BRAF* mutations associated with sporadic CRC MSI were screened, and no mutation was detected. However, at variance with CRC, *BRAF* mutations have never been detected in medulloblastomas, which thus does not exclude the sporadic nature of our MSI-positive medulloblastomas.

β-Catenin is a key player in the Wingless/Wnt signal transduction pathway that is involved in medulloblastomas, and mutations were reported previously in only 5%–9% of the cases.[57,58] Aiming to better characterize our samples and to determine if *CTNNB1* could be related to MSI status in medulloblastomas, we searched for mutations in its hotspot region but found no evidence. Although no *BRAF* or *CTNNB1* mutations were found in the hotspot region screens in these medulloblastomas, we cannot exclude the potential existence of mutations in other regions of the genes.

In conclusion, this study is the most comprehensive analysis of MSI in medulloblastomas to date. We found a total of four cases (11%) with instability, three with MSI-L, and one with MSI-H, two of which presented mutations in *MBD4* and *MRE11* MSI target genes, which have never before been reported in medulloblastomas. While further studies analyzing a larger series of both pediatric and adult medulloblastomas are warranted to assess the frequency of MSI, the present work suggests the existence of a potential novel molecular pathway in a fraction of medulloblastomas associated with the presence of MSI.

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